Comparison of T7- and lac-based systems for the periplasmic expression of human granulocyte macrophage colony stimulating factor in *Escherichia coli*

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Abstract

With the aim of the production of recombinant human granulocyte macrophage colony stimulating factor (rhGM-CSF) in the periplasmic space of Escherichia coli, the expression of hGM-CSF cDNA was examined under the regulation of a T7-based as well as a lac-based expression systems. For the efficient expression of hGM-CSF cDNA, the first five codons at the N-terminal were altered based on the E. coli major codon usage. The hGM-CSF cDNA, fused to pelB signal sequence, was expressed using the two inducible promoters. The expression analysis of the 2 recombinant plasmids were performed in the BL21(DE3) and TG1 strains of E. coli, respectively. After induction with 1mM isopropyl-ß-D-Thiogalactopyranoside (IPTG) the recombinant E. coli with T7 promoter produced hGM-CSF more efficiently than did the lac promoter. Under inducing conditions both of the recombinant bacteria allowed successful secretion of hGM-CSF into the periplasmic space. The optimal temperature for the over-expression of the recombinant protein under the T7-based system was 30°C and that of the lac regulated system was 28°C. The optimization of growth condition for the recombinant bacteria, produced in this work, provides mean for studying the function of environmental as well as genetic factors on the over-expression of recombinant proteins in the periplasmic space of E. coli.

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INTRODUCTION

Granulocyte Macrophage Colony Stimulating Factor (GM-CSF) is a 127 amino acid residue glycoprotein hormone-like CSF and one of the hematopoetic growth factors and regulator of hematopoesis that controls the production and activation of granulocytes and macrophages from progenitor cells (Welte *et al.*, 1985). This protein has been used for treatment of myelodysplastic syndrome, neutropenia and chemotherapy induced myelosuppression (Besa, 1992; Levine and Bloomfield, 1992). Consequently, a variety of potential clinical uses for rhGM-CSF are under investigation, such as prophylaxis or adjunctive treatment of infections, use as a vaccine adjuant, and use as immunotherapy for malignancies (Armitage, 1998). The large-scale production of the human GM-CSF in several heterologous gene expression systems such as mammalian cells (Lee *et al.*, 1985; Gordon *et al.*, 1985; Wong *et al.*, 1985), yeast (Miyajima *et al.*, 1986, Cantrell *et al.*, 1985) and bacterial cells (Burgess *et al.*, 1987; LaLonde *et al.*, 1989; Fischer 1994; Oloomi *et al.*, 1999) have been demonstrated so far. These preparations are not identical and differentiated mainly by degree of glycosylations (Armitage, 1998). Both mammalian and yeast cells have both advantages and disadvantages

as expression systems for producing GM-CSF in quantities as well as forms necessary for therapeutic purposes. The produced GM-CSF by the mammalian cells is appropriately folded, but it is synthesized transiently and at low level (Libby *et al.,* 1987). Yeast-derived GM-CSF, on the other hand, is produced at higher level but with the yeast–specific carbohydrate moieties (Libby *et al.,* 1987) and glycosylated to a lesser extent than the one produced in mammalian CHO cells (Armitage, 1988). However, the yeast derived rhGM-CSF is the only form of synthetic rhGM-CSF commercially available in the united state. The *E.coli* expressed hGM-CSF has enabled the large scale production of hGM-CSF, unlike the ones expressed in yeast or mammalian cells lacks carbohydrate, but exhibits higher biological activity *in vitro* than does the natural glycoprotein (Libby *et al.,* 1987). The bacterial-derived rhGM-CSF is also considered as a major synthetic rhGM-CSF.

E. coli expression system has advantage of high level production of the recombinant protein (Baneyx, 1999; Hodgson, 1993; Lee, 1996). This gram negative bacterium is well equipped to secrete proteins through the cytoplasm, and this approach has been used widely to direct heterologous proteins out of the cytoplasm. It has been particularly successful in enabeling the production, in *E. coli*, of biologically active proteins (reviewed by Hockney, 1994). Targeting the recombinant proteins to the bacterial periplasmic space has the advantages of having less onerous purification, authentic N-terminal and proper folding and suitable biological activity. Several reports have been published on the successful secretion of GM-CSF in *E. coli*, but the productions were inefficient (Libby *et al.,* 1987; Burgess *et al.,* 1987; Lalonde *et al.,* 1989; Hua *et al.,* 1994; Curless *et al.,* 1994; Fischer, 1994).

The aim of the present study was to asses the periplasmic expression of recombinant human GM-CSF in *E. coli* directed with pelB signal sequence under regulation of two promoters, namely T7 and *lac*, with regards to the issue of expression level.

MATERIALS AND METHODS

Bacterial strains, plasmids, and primers

The BL21(DE3) strain of *E. coli* (Novagen, USA) carrying a lysogenic copy of the RNA polymerase gene of bacteriophage T7 on the chromosome, and the TG1 strain (Strategene, USA) were used as expression hosts for T7-regulated and lac-regulated expression systems respectively. Clone p91023(B) (Gordon *et al.,* 1985) containing the GM-CSF cDNA was used as template for the PCR amplification of the GM-CSF cDNA. Plasmids pHEN4 (BioLabs, England) and pET26a+ (Novagen, USA) carrying pelB signal sequence coding region on the upstream of their multiple cloning sites equipped with either bacterial *lac* promoter or bacteriphage T7 promoter respectively.

An upstream primer, GM-CSF-pri3, from the beginning of the coding region of GM-CSF (5´-CATGC-CATGGCCGC**G**CC**G**GCCCGCTCGCCC-3´) with an *Nco*I cut site at its 5´ end and a downstream primer, GM-CSF-stop, (5´-GCTCCGGGATCCTTACT CCTG-GACTG-3´) from the end of GM-CSF coding region with a *Bam*HI site at its 5´ end were used for amplification of the hGM-CSF full length cDNA. The two under lines sequences are two modified codons based on the *E. coli* major codon database.

Media and culture conditions

Luria-Bertani (LB) medium (10 g/l Bacto-tryptone, 5 g/l Bacto yeast extract, and 10 g/l NaCl, pH 7.0) containing either 30 µg/ml of kanamycin for (the cultivation of) recombinant BL21(DE3) cells or 50 µg/ml of ampicillin for recombinant TG1 cells was used as culture medium. Either IPTG or lactose was used as inducers at the defined concentrations.

Enzymes and chemicals

Enzymes *Bgl*I, *Nco*I, *Bam*HI, Taq DNA polymerase and T4 DNA ligase were purchased from Roche-Germany. Taq polymerase was obtained from Cinagen-Iran. Bacto-tryptone, bacto-yeast extract and bacto-agar was purchased from E. Merck-Germany. Polyclonal rabbit antiserum raised against human GM-CSF was prepared in NRCGEB Iran. Immuno-reactive material was detected using alkaline phosphatase conjugated goat anti-rabbit antibodies (Tebsan-Iran)

DNA manipulations

Recombinant DNA techniques: DNA manipulations, such as plasmid DNA isolation, DNA digestion and sub-cloning were performed according to standard methods (Sambrook and russel, 2001). In addition to DNA digestion for the confirmation of recombinant plasmids, polymerase chain reaction (PCR) method using the specific primers was employed. Commercially prepared columns (Roche-Germany) were used for purification of DNA from agarose gel

and direct PCR product.

Polymerase chain reaction (PCR): The PCR condition for the primers GM-CSF-pri3 and GM-CSF-stop was 30 cycles of denaturing at 94°C for 1 min, annealing at 54°C for 1 min and elongation at 72°C for 1 min continued with a final elongation time of 10 min at 72°C.

Sequence analysis: The sequences of the recombinant clones were determined using ABI 373A automated sequencer. The comparison of the obtained sequences against the GenBank was performed using Blast program.

Expression analysis

Growth and inducing conditions: Isolated colonies obtained from frozen stock, grown on the selective media, were used to inoculate flasks containing 50 ml of ampicillin containing LB medium. Cells were grown at 30°C, under shaking condition, until OD_{600}=1.5 and induced by adding IPTG to a final concentration of 1 mM (or by different concentrations of lactose in case of the BL21 clones). The incubation was continued either at 30°C or 37°C for next 4 hours. The cells were then harvested and total protein pattern of samples taken at 2, 3 and 4 hours after the induction time were visualized by SDS-PAGE. The specificity of the expressed recombinant hGM-CSF was also examined by western blot method.

Osmotic shock: Periplasmic osmotic-shock fluid from hGM-CSF producing transformed *E. coli* strains was obtained by a modified method of Libby *et al.,* (1987). Briefly, on the micro-scale, 1.5 ml fermentation broth with OD_{600}=1 unit was centrifuged at 15000×g for 5 min and the pellet was collected. All the subsequent steps were carried out at 4°C. Pellets were resuspended in 15 µl of ice cold TES buffer (Tris-HCl 0.2 M, EDTA 0.5 M, Sucrose 0.5 mM) pH 8.0, shaking vigorously every second minutes. 22.5 µl of ice cold double-distilled water was added and the incubation was continued for 30 min on ice. The cells were centrifuged at 16000 ×g for 20 min. Trichloroacetic acid was added to the supernatant up to 12% of the final volume. The mixture was centrifuged at 16000 ×g for 20 min. The pellet was dissolved in sample buffer and was boiled for 5 min and saved as the periplasmic fraction for further protein analysis.

SDS-PAGE and Western blotting: SDS-PAGE was performed by a modified method described by Lammli *et al.,* (1970) and gels were stained with Coomassie brilliant blue. Electroblotting of proteins onto nitrocellulose sheets (Amersham-Pharmacia Biotech) was performed using either wet blotting procedure.

For western blotting experiment, electerophoresed proteins were transferred to a nitrocellulose membrane in a transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol) at 86 mA for overnight and probed with a polyclonal antiserum prepared against hGM-CSF. The hGM-CSF-antibody complex was then treated with horseradish peroxidase-conjugated anti-rabbit immunoglobulin and visualized using a solution of 4-chloronaphtol with hydrogen peroxidase as enzyme substrate.

rhGM-CSF expression assay: Total protein pattern of the recombinant bacteria, visualized on commassie brilliant blue stained gel, were scanned by a Beckmann mode R-112 densitometric gel scanner for estimation of rhGM-CSF ratio related to the total bacterial proteins.

Plasmid stability test

For the evaluation of the recombinant plasmids stability in the *E. coli* hosts, each clone was grown in non-selective liquid medium at 30°C for 96 hours. Equal volumes of the diluted over-night cultures were grown on both selective and non-selective LB plates at 30°C. At the same time 1 ml of the culture was used to inoculate a new shake flask containing 50 ml LB medium for cultivation during next 24 h. The ratio of the colony numbers appearing on the selective and non-selective media was considered as an estimated measurement for plasmid stability after each day during 96 hours incubation. For the BL21(DE3) clones, selective medium was prepared by adding 50 µg/ml kanamycin to the LB plates. For the TG1 clones, selective media were prepared by adding 100 µg/ml ampicillin onto LB plates.

RESULTS

Construction of recombinant plasmids

Two recombinant plasmids namely plac-pelB-hGMCSF (4275 bp) and pT7-pelB-hGMCSF (5734 bp) were constructed similarly by inserting a PCR product containing a human GM-CSF cDNA, in the *Nco*I/*BamH*I restriction sites, next to a pelB signal

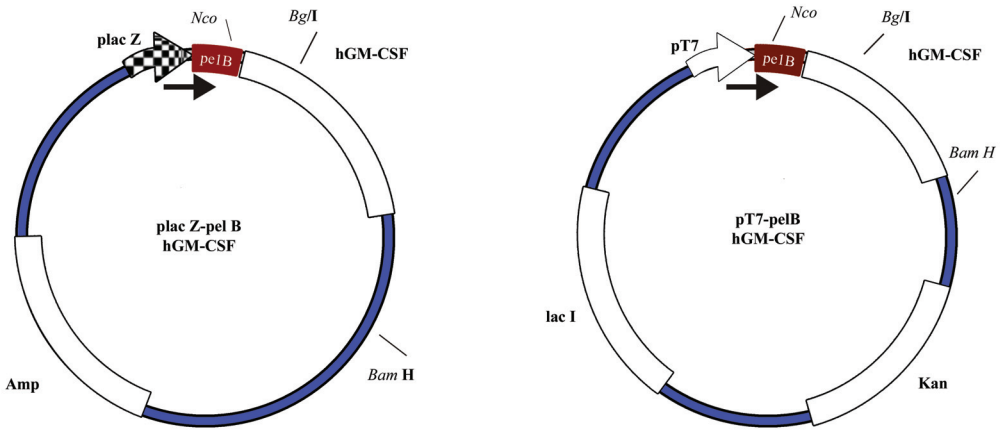

Figure 1. The physical map of the pT7-pelB-hGMCSF and plac-pelB-hGMCSF constructs

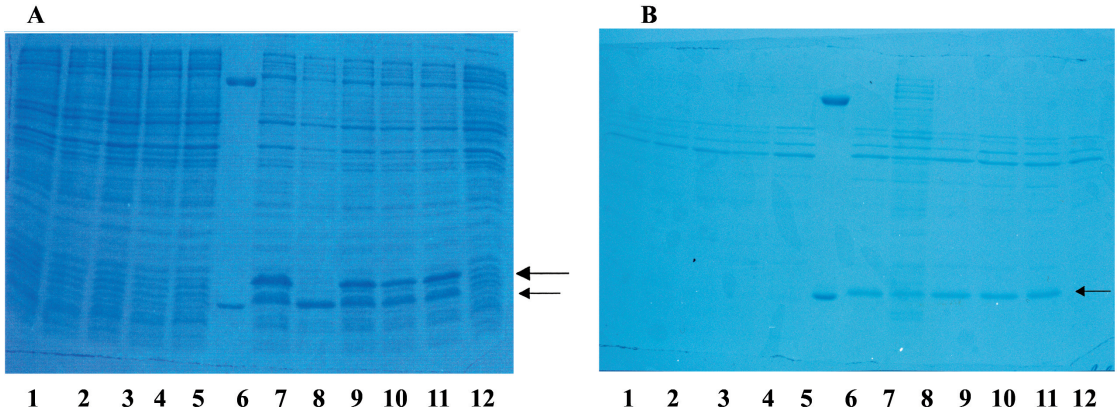

Figure 2. Identification of rhGM-CSF expressed by the pT7-pelB-hGMCSF construct in the recombinant BL21(DE3) strain of *Escherichia coli* by SDS-PAGE analysis 3 hours after induction. Panel A: The total protein pattern of the bacteria. Panel B: The periplasmic proteins of the bacteria separated after osmotic shock. Lanes 1 to 5: Samples taken from the recombinant bacteria, grown for three hours under un-induced conditions. Lanes 6: Standard human GM-CSF. Lanes 6-11: samples taken from the recombinant bacteria, 3 hours after induction with IPTG. Lane 12: BL21(DE3) carrying a pET26(b+) without insert grown in similar inducing condition. The locations of mature and unprocessed recombinant proteins are indicated by thin and thick arrowheads respectively.

sequence coding region and transformed into the TG1 strain and BL21(DE3) strains of *E. coli* respectively. Using the *Nco*I restriction site for joining of pelB and GM-CSF coding region caused an insertion of two additional amino acids (Ala and Met) at the C terminal of the signal peptide in the junction site, that apparently did not disturb the processing of the signal peptide (see below). Considering the only *Bgl*I site in the GM-CSF cDNA (Fig. 1), the *Bgl*I digested pattern of the recombinant plasmids confirmed the cloning of the cDNA in both vectors (results not shown). The recombinant bacteria were subjected to further studies and subsequent expression analysis.

rhGM-CSF expression analysis

Total protein patterns of the 5 isolated BL21 clones, containing pT7- pelB- hGMCSF construct taken 3 hours after induction with IPTG observed by SDS-PAGE as well as western blot analysis showed an over-expression of a 14 KD protein (Fig. 2). This was further confirmed by its reaction with the rabbit serum directed against hGM-CSF. The size and specificity of

the recombinant protein was comparable to the standard hGM-CSF. Comparison of the periplasmic and total protein pattern shows that the recombinant BL21(DE3) bacteria harboring pT7-pelB-GM-CSF secreted detectable amount of a unique polypeptide band, in the periplasmic fraction of the bacterial protein. The larger protein which is thought to be unprocessed pelB::hGMCSF precursor exclusively associated only the total as well as cytoplasmic proteins but not the periplasmic fraction of the recombinant bacteria. A longer incubation time after induction may increase the level of the mature hGM-CSF in the preiplasmic space. The expression assay for the recombinant protein (mature plus precursor forms of rhGM-CSF) shows that the highest expression rate occurs in the presence of IPTG at 30°C.

The total protein pattern of the recombinant TG1 bacteria (carrying the plac-pelB-hGM-CSF constructs), 3 hours after induction with IPTG, was analyzed by SDS-PAGE and followed by western immunoblot analysis. A 14 KD product co-migrated with standard hGM-CSF and a larger protein, exclusively associated with the recombinant clones were only detectable by the rabbit serum directed against hGM-CSF (Fig. 3). Results obtained from western blotting analysis suggest possible degradation of the recombinant GM-CSF produced by these clone set (Fig. 3). The optimal temperature for the highest expression level of the recombinant hGM-CSF under the lac regulated system in the presence of IPTG was 28°C. However several attempts to optimize the growth as well as inducing conditions on this clone did not lead to the over-expression of rhGM-CSF.

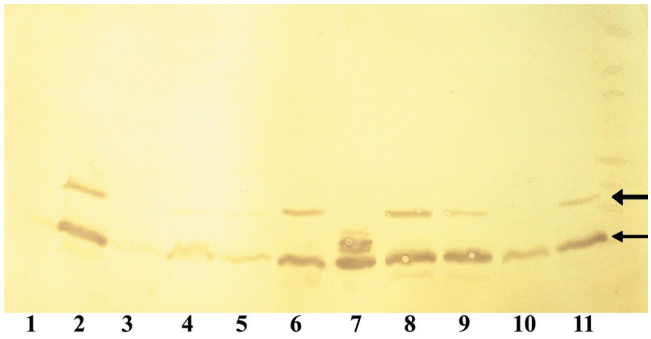

Figure 3. Identification of rhGM-CSF expressed by the plac-pelB-hGMCSF construct in the total protein of the recombinant TG1 strain of *Escherichia coli* by western blot analysis 3 hours after induction. Lane 1: TG1 bacteria carrying a pHEN4 plasmids without insert grown in similar inducing condition. Lanes 2-6 and 8-11: Samples taken from the total protein of 9 isolated clones, grown for three hours under un-induced conditions. Lane 7: Standard human GM-CSF. The locations of mature and unprocessed recombinant proteins are indicated by thin and thick arrowheads respectively.

Lactose induction

We have also studied the inducing activity of the natural inducer (lactose) for the induction of rhGM-CSF expression in *E. coli* under the two examined regulatory systems, using different concentrations of lactose, from 0.02% to 2%. Only the T7-based expression system in the BL21 strain was activated in response to lactose induction (Fig. 4). Among different concentra-

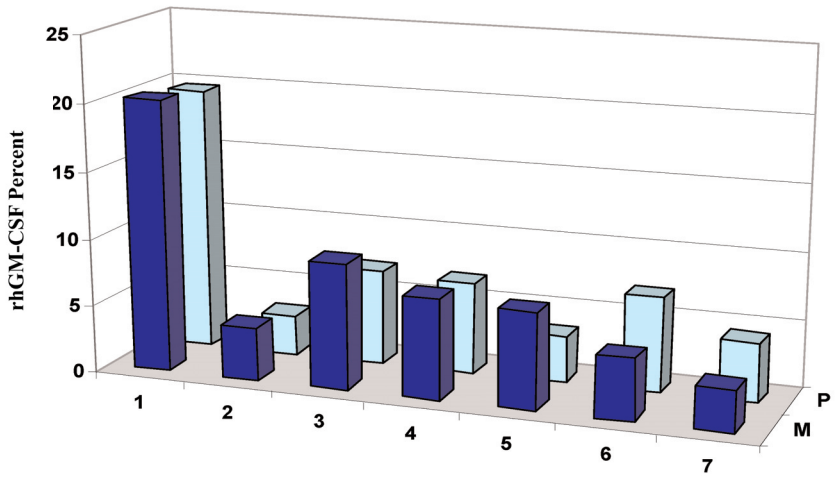

Figure 4. Estimation of mature (M) and precursor (P) forms of rhGMCSF produced in the pT7-pelB-hGM-CSF clones 3 hours after induction by different concentrations of lactose comparing to that of IPTG induction. The numbers in X-axis correspond to inducers as the following: **1**: 1mM IPTG. **2**: 2% lactose. **3**: 1% lactose. **4**: 0.5% lactose. **5**: 0.1% lactose. **6**: 0.05% lactose. **7**: 0.01% lactose.

tions, the lactose concentration of 10 g/l and cell optical density of 1 to 1.5 at 590 nm appeared to be conditions for the highest level of priplasmic expression of human GM-CSF that was less than 10% of the total cell protein. Comparing to the highest expression level occurred after IPTG induction (20% of the total cell protein) the efficiency of lactose induction was very low. Some of our data (not shown here) suggests that the elevation in the lactose inducing time may increase the expression level.

Plasmid stability tests

The plasmids plac-pelB-GMCSF and pT7-pelB-GMCSF are stably maintained in their hosts, TG1 and Bl21(DE3), respectively as judged by comparing the number of colonies on LB-plates with and without antibiotic selection. Thus, the lower production of rhGM-CSF in the case of plac-pelB-GM-CSF clone is most likely due to it's lower promoter activity or protein instability rather than the plasmid instability.

DISCUSSION

When the mRNA of heterologous target gene is over-expressed in *E. coli*, differences in codon usage can impede translation because of demand for rare tRNA. Insufficient tRNA pools may stall translation and terminate it prematurely, or cause frame shifting or amino acid misincorporation. Therefore, codon usage is considered a potential factor affecting product yield and implies that heterologous genes enriched with codons that are not used frequently by *E. coli* may not be expressed efficiently in *E. coli* (Makrides, 1996). However, it appears that the presence of rare codons near the 5´ end of a transcript affects translational efficiency. One of strategies, developed to minimize the effects of preferential codon usage in *E. coli* relies on genetically altering rare codons in the target gene without modifying the encoded protein product. Alternatively, the intracellular tRNA pool can be expanded by co-expressing genes that encode rare tRNAs (Hannig and Makrides, 1998). Based on the first approach, in the present work, the first five codons on the hGM-CSF cDNA (GCA CCC GCC CGC TCG), which code for alanine, proline, alanine, argenine and serine, respectively, were subjected for the minimization of the effect of preferential codon usage in the recombinant plasmids constructed in this work. Accordingly, using site directed mutagenesis, the codons for the first two amino acids, alanine and proline, were changed to GC**G** and CC**G** respectively which are among the major codons in *E. coli*. The next three codons in the row, which were among the major codons in *E. coli*, were left intact.

The protein patterns obtained from the whole cell, cytoplasm and periplasm, prepared by osmotic shock of the two recombinant bacteria constructed in this work, show that a major part of the expressed rhGM-CSFs (pelB::hGM-CSF) are processed and directed into the periplasmic spaces of the both recombinant bacteria. The expression level of rhGM-CSF under the regulation of the T7 promoter was increased to about 20% of the total bacterial protein that is much higher comparing to the hGM-CSF expression under the lac promoter. The optimal temperature for recombinant bacteria in the presence of inducer was 28°C and 30°C under the lac and the T7/lac systems respectively. Although the expression level is higher in the T7-based system, the relative amount of the mature GM-CSF was less than that of the lac-based recombinant bacteria that indicates in higher efficiency of processing in the lac regulated clones. Low efficient processing of human GM-CSF in the *E. coli* periplasmic expression system could occur as a result of the formation of inclusion bodies (Ibs) of the recombinant protein or unefficient function of secretion system. Relatively higher ratio of mature GM-CSF observed in the periplasmic space of the plac-pelB-GM-CSF containing bacteria may be correlated to the lower expression level of the recombinant protein that give advantages to the cell to complete the processing of over-expressed recombinant proteins before inclusion body formation. Secretion of proteins is rarely 100% efficient and several strategies for improved translocation of proteins to the periplasm have been reported (Makrides, 1996). Therefore any solution that enhances the solubility of the protein and reduces the inclusion body formation may increase the signal peptide processing of the periplasmic directed proteins. Some of these solutions are the over-production of signal peptidase I, a reduction in protein expression levels in order to prevent the overloading of the translocation machinery.

Reduced fermentation temperature has been partially successful with a number of proteins. Moreover, the positive effect of the co-expression of some proteins including those of sec translocation pathway as well as some molecular chaperonines which are involved in maintaining protein solubility in cytoplasm is well

documented for the enhancement of recombinant proteins in *E. coli* (Collier, 1994; Diamant *et al.,* 2000; Fraipont *et al.,* 1994; Pogliano and Beckwith 1994). The effect of chaperonines on the efficiency of the periplasmic expression of hGM-CSF was shown by Berges and his colleagues in a combined approach (1996). They showed that increasing of the perplasmic fraction of the recombinant protein by the overproduction of SecB or DnaK plus DnaJ depends on the signal peptide-heterologous protein-chaperone association involved. Considering the various numbers of the key-factors on the periplasmic expression level, by taking advantage of combined approaches, the periplasmic expression of a number proteins in *E. coli* were significantly improved (Weikert *et al.,* 1996).

As it was expected, the TG1 recombinant bacterium, produced in this study, due to the absence of functional *lacY* gene (codes for lactose permease) did not respond to lactose induction. The lactose permease gene product is essential for the import of lactose to the cell. To use lactose as an inducer, *E. coli* must first transport the molecule into the cell (using a lactose permease, the product of the *lacY* gene). Once inside, lactose binds to the repressor protein, causing it to change into a conformation that can no longer bind to DNA, therefore allowing RNA polymerase to transcribe the genes. In this way, the gene under regulation of T7/lac based system is only transcribed when lactose (or lactose analog) is present. Optimization of growth condition for the recombinant bacteria presented in this work and the use of lactose as inducer (in the case of the T7-based clone) or considering a combined approach such those mentioned above are among alternative choices for the next steps in the production of rhGM-CSF. The effect of modifications can be rationalized in terms of reducing the rate at which newly synthesized protein is released from the protein-synthetic machinery into the cell cytoplasm or by altering the milieu in which the protein must fold.

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